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GENETIC ANALYSIS OF DOUBLE KNOCK-OUT AND DOUBLE OVEREXPRESSION OF ALDEHYDE DEHYDROGENASE (ALDH) MUTANTS FOR DROUGHT TOLERANCE IN *ARABIDOPSIS THALIANA*

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Abstract

Different *aldehyde dehydrogenase* (*ALDH*) knock-out mutants and transgenic *Arabidopsis thaliana* overexpressing *ALDH* genes are analysed in this study in order to establish the genotype of each independent plant and to elucidate the functional involvement of *ALDH* genes in plants response to abiotic stress. Approximately, hundred *ALDH* double knock-out plants are analysed by PCR amplification using two different pairs of primers for *ALDH*, out of 33 analysed plants three single knock-out plants for *ALDH*4, out of 42 analysed plants twelve single knock-out plants for *ALDH*5 and one double knock-out for *ALDH*3 and *ALDH*5 are found in total of 75 analysed plants. In addition, double overexpressing plants for *ALDH*3I1 and *ALDH*7B4 genes, are identified in the same plant. Out of 24 plants analysed, 6 plants are detected to have both *ALDH* transgenes. Further, the accumulation of *ALDH*3I1 and *ALDH*7B4 proteins are detected in the double overexpressing plants using respective *ALDH* antibody. The follow-up work is to carry out the physiological and biochemical characterization of these plants under different abiotic stress conditions.

Keywords: *Aldehyde dehydrogenase*, Stress tolerance, Double knock-out, Double overexpressing

1. Introduction

Oxidative stress is thought to be one of the major causes of cellular damage and cell death following abiotic stress such as drought stress (Bartels, 2001; Mittler, 2002; Ramanjulu and Bartels, 2002; Hou and Bartels, 2015). Aldehydes and their intermediates are common by-products of a number of metabolic pathways (Zhang *et al*., 2012). The removal of aldehydes and their intermediates is essential for cellular survival. *Aldehyde dehydrogenase*s (*ALDH*s) catalyse the oxidation of toxic aldehydes to their non-toxic corresponding carboxylic acids. Various distinct *ALDH*s have been

studied and widely characterized especially in humans and in yeast (Lindahl, 1992; Yoshida *et al*., 1998; Navarro-Aviño, 1999). Understanding the processes by which plant-*ALDH* activities limit the cellular damage caused by toxic aldehydes may represent a critical protective strategy for surviving osmotic and even oxidative stress in plants. For more than two decades, the research in understanding the mechanism of various environmental stress responses is depending on the alleles of specific genes for stress tolerance in *A. thaliana* (Bressan *et al*., 2013).

In plants five different *ALDH* genes, all belonging to the mitochondrial and cytosolic class 1/2 isozymes have been analyzed. In order to investigate the physiological role and possible involvement in stress response, the genes encoding the enzyme *ALDH*s are suppressed or mutated by *Agrobacterium* mediated T-DNA insertions (Bechtold, 1993; Bechtold and Pelletier, 1998; Bent, 2000; Szabados *et al*., 2002) and overexpressed by expression of a transgene under the transcriptional control of the CaMV 35S promoter. Using the combination of loss-of-function mutants and transgenic plants, the role of the *ALDH* in plant stress responses can be studied. Plants, in which the genes coding for a single *ALDH* enzyme are disrupted (*ALDH3*, *ALDH4*, *ALDH5* or *ALDH6*) and are called single knock-out plants. In order to investigate the function of gene in stress tolerance, the single knock-out plants are crossed with each other to produce double knock-out plants. Simultaneously, plants that overexpress the *ALDH*3 and *ALDH*6 genes are crossed with each other to obtain double overexpressing plants (two different overexpressing *ALDH* genes present in the same plant). The analysis in this study includes the identification of double knock-out mutants (homozygous plants) that suppress the function of the two specific *ALDH* genes and the selection of the overexpression of *ALDH*3I1 and *ALDH*7B4 in the same plant by using specific primers (Fig. 1).

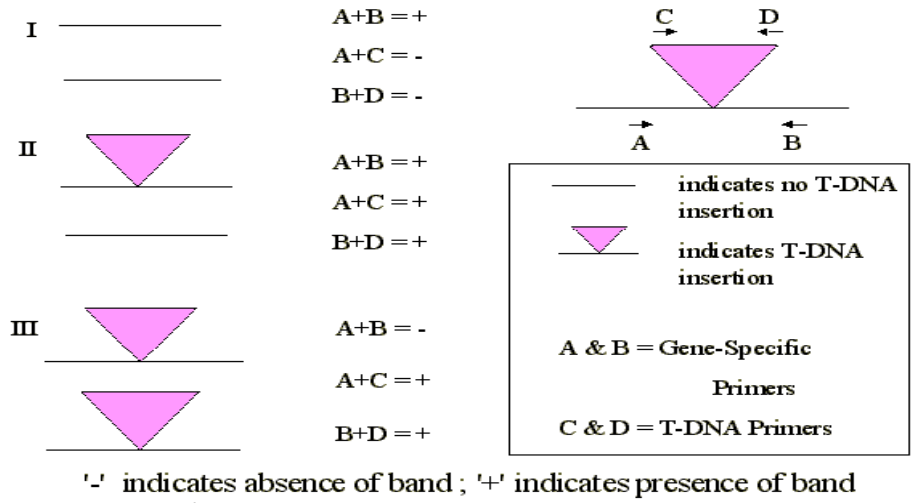

Figure-1: Diagrammatic representation of the primers involved in PCR analyses for heterozygous knock-out

plants.

The knock-out mutants of *ALDH*3I1 (old nomenclature – *ALDH*3) consist of a T-DNA insertion (Koncz *16843) in the second exon of the coding sequence. The length of the coding sequence is about 1653bp. The knock-out mutants of *ALDH*3H1 (old nomenclature – *ALDH*4) consist of a T-DNA insertion (Koncz *22184) in the first exon of the coding sequence.

The length of the coding sequence is about 1455bp. The knock-out mutants of *ALDH*3F1 (old nomenclature – *ALDH*5) has a T- DNA insertion (SALK *091250) in the third exon of the coding sequence. The total length of the coding sequence is about 1452bp. The knock-out mutants of *ALDH*7B4 (old nomenclature – *ALDH*6) contain a T- DNA insertion (SALK *143309) in the non-translated region. The length of the coding sequence is about 1527 bp.

The present study involved objectives such as isolation of genomic DNA from the crossed Arabidopsis thaliana lines, amplification of *ALDH* genes to identify those positive plants that either are mutated in two specific *ALDH* genes and over expressed *ALDH3* and *ALDH7* and ultimately, the detection of positive double-mutant plant and double over expression lines through immunological techniques using specific antisera.

2. Materials and methods

2.1. Plant Material and Genomic DNA extraction

Plant material was collected from the inter-crossed two different single knock-out plants and double overexpressing plants (Kirch *et al*., 2005). The leaf material is used as the explants for genomic DNA extraction. Plant genomic DNA extraction was performed with urea treatment.

Genomic DNA Primer Sequences for Double Knock-out plants are as follows: The primers for ALDH3 (Koncz) are Forward Primer 5’ TTGCCAAGGGTTTTTCTCCTGCCAG 3’and Reverse Primer 5’ TAAGAT CCGCGTCCCCTGAAAAGCT 3’; ALDH4 (Koncz) are Forward Primer 5’ ATCGGCGGAAGCGA GTAATTTGGTG 3’ and Reverse Primer 5’ TATGGCGGATACCTGACGGCT GAATC 3’; ALDH5 (SALK) are Forward Primer 5’ GAAGCCATGGAAGCTATGAAGGAGAC 3’ and Reverse Primer 5’ GTCT CTGTCTCTCACTTTCCCCCTT 3’; ALDH6 (SALK) are Forward Primer 5’ CATACGAGGATGATCGTG GCAATGTC 3’ and Reverse Primer 5’ TCACCTCTCTTAGGAGC CGTAACCT 3’; T-DNA Primers are FISH 1 5’ CTGGGAATGGCGAAGTCAAGGCATC 3’ and FISH 2 5’ CAG TCATAGCCGAATACGGTC TCCA 3’.

2.2. Polymerase chain reaction for the analysis of double Knock-out plants

PCR was performed for the double knock-out plants with gene-specific primers and T-DNA primers for *ALDH*3, *ALDH*4, *ALDH*5 and *ALDH*6 respectively (Table 1). PCR for observing the double knock-out plants engage two pairs of primers such as gene-specific and T-DNA primers.

Table-1: The genotype names of heterozygous double knock-out plants obtained after crossing of single knock-out plants.

M/F	*ALDH*3	*ALDH*4	*ALDH*5	*ALDH*6
*ALDH*3	-	A1	B2/B3	C1/C2
*ALDH*4	Z1/Z3	-	D3/D4	E1/E3
*ALDH*5	Y3/Y4	W2/W3	-	F1/F3
*ALDH*6	X2/X4	V3/V4	U1/U2	-

2.3. Polymerase chain reaction for the analysis of double over-expression plants

PCR was performed for the over expressing lines of *ALDH*3I1 and *ALDH*7B4 with the corresponding forward and reverse primers.

2.4. Protein extraction for double over-expressing plants and SDS-PAGE

Protein extraction was performed using the Laemmli Buffer from the leaf explants. The separating gel was made of 12% (w/v) acrylamide and the stacking gel was made of 4% (w/v) acrylamide. Protein detection on the membrane was performed by ponceau staining [0.2 % (v/v)] ponceau S in 3% (w/v) TCA before carrying out the antibody detection of specific protein synthesis.

2.5. Protein blot and antibody detection

The separated protein samples were transferred from the gel onto a Protran nitrocellulose membrane BA 85-membrane (Schleicher and Schuell, Dassel) using a protein blot transfer buffer (PBTB) as described by (Towbin *et al*., 1979) at 100 mA for overnight. The protein accumulation was detected by using specific primary antibodies against ALDH3I1 and ALDH7B4 (in the ratio of 1:500 and 1:1000 respectively) produced from immunized rabbits with the fusion proteins with

GST (Amersham Pharmacia Biotech, Freiburg). The secondary antibody (in the ratio of 1:10,000) used is anti-rabbit IgG horse-radish peroxidase-linked antibodies (Sigma). The protein detection was done using ECL plus Western blotting detection kit (ECL-Amersham Pharmacia Biotech). The program used for the ECL detection is Image Reader LAS - 1000 Lite version 1.3.

3 Results and discussion

Limited characterizations have been carried out on the corresponding plant-*ALDHs*. *ALDH* represent a group of enzymes, which may play a role in stress relevant detoxification processes. It has been reported that various plant-*ALDH* transcripts accumulate in response to environmental stresses (Barclay *et al*., 1994; Kirch *et al*., 2001). ALDHs have been studied in various organisms from bacteria to mammals. The shortages of food may arise due to the minimized production ability of the crops (Hasthanasombut *et al*., 2010). The economically important crop plants such as potato, tobacco and rice are prone to drought and saline stress. Therefore, the development of resistance to drought and salinity may enhance the production of crops worldwide (Zhang *et al*., 2011; Zhang *et al*., 2013). The first identified *ALDH* gene (Rf2) represents a maize nuclear restorer gene of cytoplasmic male sterility (Cui *et al*., 1996). Recently, two rice acetaldehyde-oxidizing *ALDHs*, *ALDH1a* and *ALDH2a*, were characterized. *ALDH1a* is a cytosolic enzyme, while *ALDH2a* seems to be localized in mitochondria (Li *et al*., 2000).

As discussed in the Table 2,3 & 4 out of 61 analyzed plants thirteen **s**ingle knock-out plants for *ALDH*3, out of 33 analyzed plants three single knock-out plants for *ALDH*4, out of 42 analyzed plants twelve single knock-out plants for *ALDH*5 and one double knock-out for *ALDH*3 and 5 were found in total of 75 analysed plants. PCR results for the knock-out plants showed the genotype of the plants. The presence of band with *ALDH* gene-specific primers indicates the absence of T-DNA insertion. The presence of band with *ALDH* gene-specific primers plus T-DNA primers indicates the T-DNA insertion within the corresponding *ALDH* gene. The gene-specific primers are unable to amplify the T-DNA sequences resulting in the absence of band having insertions. The absence of band both with gene-specific primers and *ALDH* gene-specific primers with the T-DNA primers may be due to either the less concentration of DNA or the loss of binding of the primers to the plant *ALDH* gene sequence.

As shown in Figure 2 and 3, the single knock-out plants shows the presence of band with the primer pair containing both *ALDH* gene-specific primers and T-DNA primers with respect to one of the either *ALDH* genes of the parent. The

double knock-out plants show the presence of band with primer pair containing both *ALDH* gene-specific primers and T-DNA primers for both the *ALDH* genes of the parent as shown in Fig. 2 and Fig. 3. The generation of the double knock-out plant is expected to give morphological identification of the plants and to study the molecular role of the *ALDH* genes in the stress tolerance. The identification of more double knock-out will be performed with the physiological characterization of the respective plants.

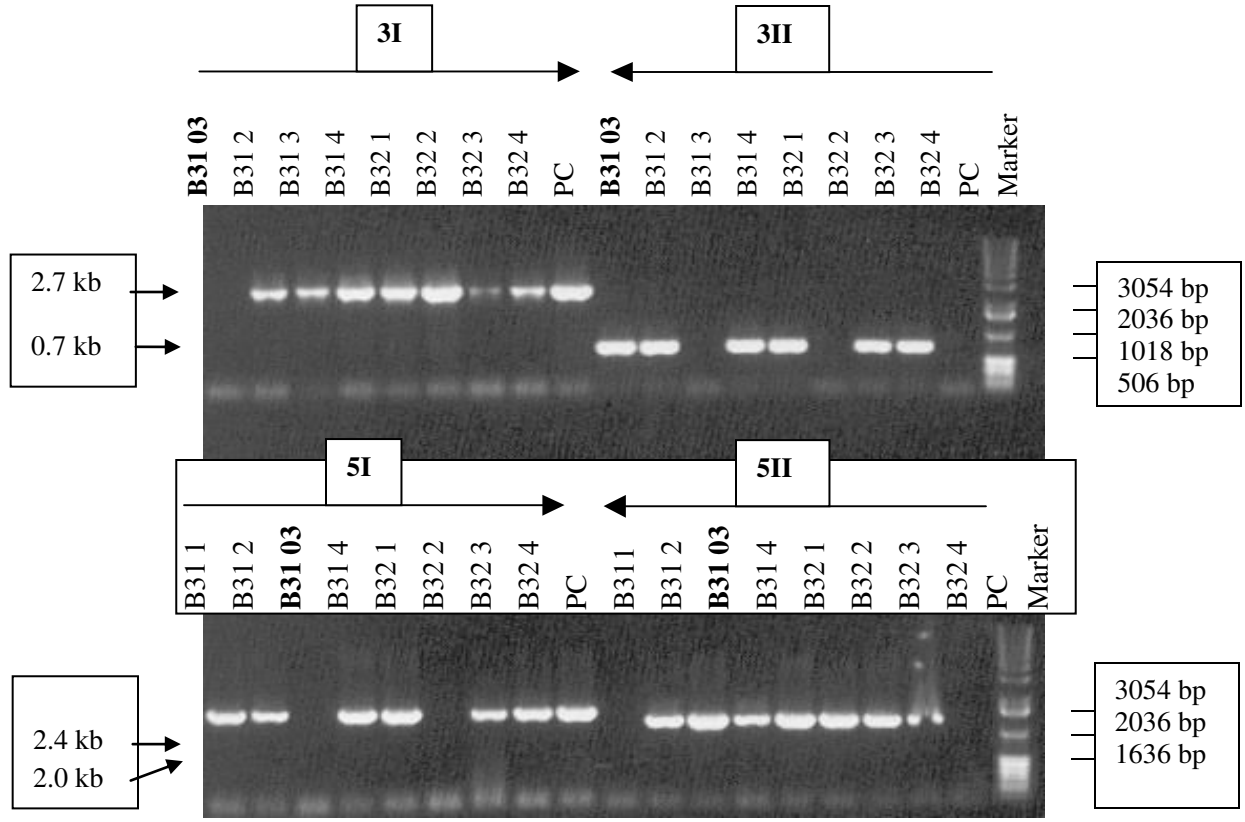

Figure-2: PCR for the analysis of double knock-out plants – *ALDH*3 and *ALDH* 5. The bold indicates the double knock-out plant. PC-Positive Control (wild-type DNA).

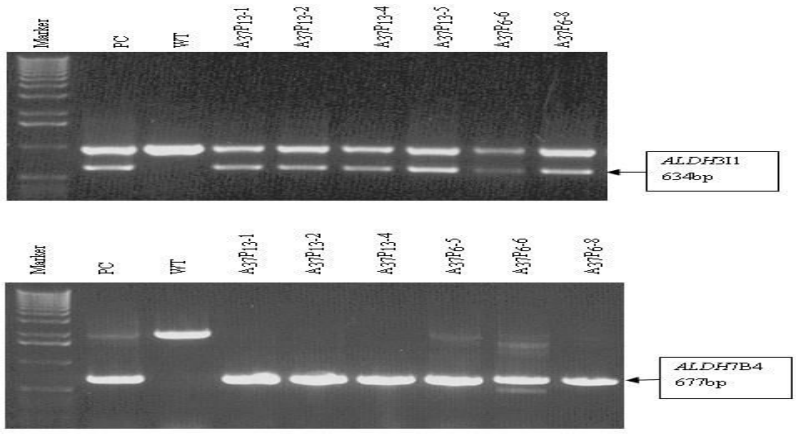

Figure-3: PCR Results for Double Overexpressing Plants of *ALDH*3I1 (a) and *ALDH*7B4 (b) genes.The arrows indicate the size of amplified *ALDH* genes using specific primers.

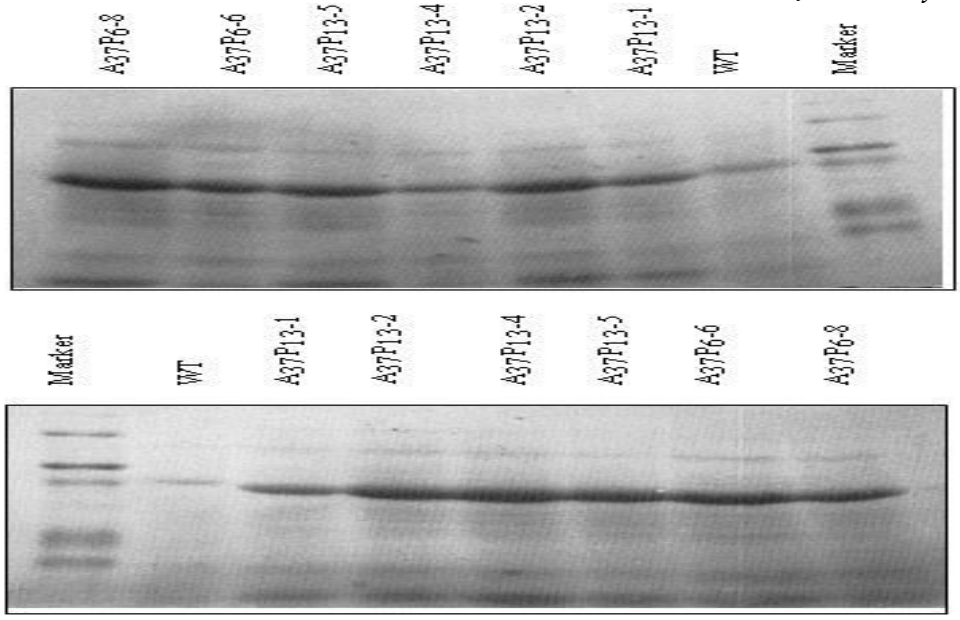

Figure-4: Ponceau staining of the protein blot analysis (a, b) of double over expressing *ALDH*3I1 and *ALDH*7B4 plants. The strong bands indicate the rubisco protein. A37 indicates *ALDH* 3 and *ALDH*7; Px indicates Plants numbering.

In addition, *ALDH*3I1 and *ALDH*7B4 gene specific forward and reverse primers were used to identify the independent double over expressing plants by amplifying both *ALDH* transgenes in the same plant. Out of 24 plants analysed, 6 plants were detected to have both *ALDH* transgenes and named as double over expressing plants as shown in Figure 3. The accumulation of *ALDH*3I1 and *ALDH*7B4 proteins were analysed in the double over expressing plants using respective *ALDH* antibody as shown in Fig. 4.

PCR results for untreated double over expressing transgenic plants revealed the expression of transgene for both *ALDH*3I1 and *ALDH*7B4 genes. Previous studies have shown that *ALDH*3I1 and *ALDH*7B4 are strongly induced under salt stress (200mM NaCl), dehydration and exogenous application of ABA (Kirch, 2004). The survival rate of the double over expressing plants is higher than the wild type plants. The over expression of *ALDH*3I1 and *ALDH*7B4 genes in transgenic plants were proven to significantly reduce the level of lipid peroxidation under drought and salt stress. The protein blot analysis of the double over expressing plants shows the expression of both *ALDH*3I1 and *ALDH*7B4 as expected. The presence of unspecific bands in the protein blot analysis of *ALDH*3I1 shows the non-specificity of the primary antibody raised by the immune system of rabbit against the respective plant proteins, *ALDH*3I1 and *ALDH*7B4 respectively. The abiotic stress tolerance is proven to be enhancing through over expression of some *ALDH* genes (Stiti *et al.*, 2011).

Table-2: PCR Results for knock-out plants *ALDH*3 and *ALDH*5.

HZ - Homozygous; HTZ - Heterozygous; SKO - Single knock-out;

DKO - Double knock-out. 'X' - Presence of band and '-'Absence of band in PCR.

S. No.	Name of Plants	*ALDH* 3		*ALDH* 5		Genotype of Plants								
						ALDH 3				*ALDH* 5				
		Gene specific primers	Gene specific and T-DNA primers	Gene specific primers	Gene specific and T-DNA primers	HZ	HTZ	HTZ/KO	SKO	HZ	HTZ	HTZ/KO	SKO	DKO
1	B311	-	X	X	X			X	X	X				
2	B312	X	X	X	X		X				X			
3	B313	X	-	-	X	X						X	X	
4	B314	X	X	X	X		X				X			
5	B321	X	X	X	X		X				X			
6	B322	X	-	-	X	X						X	X	
7	B323	X	X	X	X		X				X			
8	B324	X	X	X	X		X				X			
9	B331	X	-	-	X	X						X	X	
10	B332	X	-	X	-	X				X				
11	B333	X	X	X	X		X				X			
12	B334	X	X	X	X		X				X			
13	B341	-	-	X	X	X					X			
14	B342	-	X	X	X			X	X		X			
15	B343	X	X	X	-		X			X				
16	B344	X	X	-	X		X					X		
17	B351	-	X	X	X			X	X		X			
18	B353	-	X	-	-			X	X	X				
19	B361	-	X	X	-			X	X	X				
20	B362	-	X	X	-			X	X	X				
21	B363	X	X	X	-		X			X				
22	B364	X	X	X	-		X			X				
23	B371	X	-	-	-	X				X				
24	B372	X	X	X	-		X			X				
25	B373	X	X	X	X		X				X			
26	B374	-	X	X	-			X	X	X				
27	B381	-	X	X	-			X	X	X				

28	B382	X	X	X	X		X			X				
29	B383	X	-	-	X	X					X	X		
30	B384	X	X	-	X		X				X	X		
31	B391	X	X	-	X		X				X	X		
32	B392	-	X	X	-			X	X	X				
33	B393	-	X	X	-			X	X	X				
34	B394	X	X	X	X		X			X				
35	B3101	X	X	-	X		X				X	X		
36	B3102	X	X	-	X		X				X	X		
37	B3103	-	X	-	X			X			X		X	
38	B3104	X	X	X	X		X			X				
39	B3111	X	X	-	X		X				X	X		
40	B3112	-	X	X	X			X	X	X				
41	B3113	X	X	X	X		X			X				
42	B3114	X	X	-	X		X				X	X		

Table-3: PCR Results for the Knock-out Plants for *ALDH*3 and *ALDH*4.

HZ - Homozygous; HTZ - Heterozygous; SKO – Single knock-out;

DKO - Double knock-out. 'X' - Presence of band and '-'Absence of band in PCR.

S.No.	Name of Plants	*ALDH* 3		*ALDH* 4		Genotype of Plants								
						ALDH 3				*ALDH* 4				
		Gene specific primers	Gene specific and T-DNA primers	Gene specific primers	Gene specific and T-DNA primers	HZ	HTZ	HTZ/KO	SKO	HZ	HTZ	HTZ/KO	SKO
1	A111	-	X	X	X			X	X		X		
2	A112	X	-	X	-	X				X			
3	A113	X	X	X	-		X			X			
4	A114	X	X	X	X		X				X		
5	A121	X	-	X	X	X					X		
6	A122	X	X	X	X		X				X		

7	A123	X	X	-	X		X				X	X
8	A124	X	X	X	-		X			X		
9	A131	X	X	X	X		X				X	
10	A132	X	X	-	-		X			X		
11	A133	X	-	X	X	X					X	
12	A134	X	-	X	-	X				X		
13	A141	X	-	X	-	X				X		
14	A142	X	-	X	X	X					X	
15	A151	-	X	X	X			X	X		X	
16	A152	X	-	X	-	X				X		
17	A191	-	X	X	-			X	X	X		
18	A192	X	X	-	X		X				X	X
19	A193	X	X	X	-		X			X		

Table-4: PCR Results for the Knock-out Plants of ALDH4 and ALDH6.

HZ - Homozygous; HTZ - Heterozygous; SKO – Single knock-out;

DKO - Double knock-out. ‘X’ - Presence of band and ‘-‘Absence of band in PCR.

S.No.	Name of Plants	*ALDH* 4		*ALDH* 6		Genotype of Plants							
						ALDH 4				*ALDH* 6			
		Gene specific primers	Gene specific and T-DNA primers	Gene specific primers	Gene specific and T-DNA primers	HZ	HTZ	HTZ/ KO	SKO	HZ	HTZ	HTZ/ KO	SKO
1	V311	X	X	X	X		X				X		
2	V312	-	-	X	X	X					X		
3	V321	X	-	X	X	X					X		

4	V322	X	X	X	-		X			X		
5	V323	-	X	X	X			X	X		X	
6	V324	X	X	X	X		X				X	
7	V331	X	X	X	X		X				X	
8	V332	X	-	X	-	X				X		
9	V334	X	-	X	X	X					X	
10	V341	X	X	X	X		X				X	
11	V342	X	X	X	-		X			X		
12	V343	X	X	X	X		X				X	
13	V351	X	X	X	X		X				X	
14	V352	X	-	X	X	X					X	

4. Conclusions

The attempt to generate the double knock-outs from single knock-out mutant plants is expected to provide morphological identification of the tolerant plant and the molecular role of that particular gene in the stress tolerance can be determined. This work represents a major contribution in understanding the molecular function of the aldehyde dehydrogenases and their potential to confer osmotic and oxidative stress tolerance in higher plants.

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